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THE SIZES OF THE INFECTIVE PARTICLES IN THE MOSAIC DISEASE OF TOBACCO

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INTRODUCTION

The present investigation is one of a series of studies in progress or proposed with the idea of gaining further information concerning the constitution and behavior of the causal agency in the mosaic disease of tobacco or other mosaic diseases. We have undertaken this work with the feeling that all facts tending to throw new light upon any physical or chemical characteristic of the agency concerned might be helpful in the study of some or all mosaic diseases, and likewise, perhaps, in the study of ultramicroscopic agencies causally related to certain human and other animal diseases. The term agency rather than organism is employed because it is hoped to avoid any possible prejudice to the direction in which such research may lead. It is distinctly felt that any assumption tacitly ascribing such diseases, because infectious, to organisms of the known or usual types may serve in the end to restrict rather than broaden the investigation. The term "virus" will be used in this paper interchangeably with agency.

It is, we believe, more frequently stated that the active agency

in the mosaic disease of tobacco is a filterable virus, the "contagium vivum fluidum" of Beijerinck ('98). Confirmation of Beijerinck's porous filter experiments is not lacking. On the other hand, the agency in this disease has been found to be held back, or non-filterable, when certain filters are employed. Experiments establishing the last-mentioned fact have been contributed by the work of Allard ('16) and also for the cucumber mosaic by Doolittle ('20). All too frequently, it would seem, our knowledge respecting the particles or individuals of the so-called filterable organisms has been chiefly the fact of the passage of infective particles through some bacteriological filter, more particularly the Chamberland or the Berkefeld, with no particular effort to effect a more precise standardization of both the filters permitting the passage of such particles and of those filters restraining them, so as to permit a more definite measurement of the particles concerned.

In this work some of the methods of ultrafiltration have been employed. In general, the method or technique of the experimentation may be divided into 3 phases: (1) filtration (or diffusion) of diseased juice through various ultrafilters, (2) inoculation of healthy plants with the filtrates obtained, and (3) the standardization of the filters by a determination of their capacity to permit or prevent the passage of colloidal particles of known, or approximately known, sizes.

PRELIMINARY EXPERIMENTS

Preceding a discussion of the later work under 3 headings corresponding to the 3 phases, or aspects, above noted, it seems well to report certain preliminary data, secured during the previous year, which led to the more definite formulation of the chief experimental work reported in this paper. The preliminary experiments consisted of: (1) a filtration test of infected juice through a Livingston spherical atmometer cup, (2) filtration through layers of 1.5 and 3.0 per cent agar, (3) diffusion through Schleicher and Shüll parchment diffusion shells.

In these preliminary experiments with the atmometer the filter cup was partially filled with the juice, and suction was

then applied, a colorless filtrate being obtained with a pressure of about .5 atmosphere or more. The agar filtrates were obtained in the first instance by covering a Buchner funnel with filter-paper and then pouring on and congealing a layer of the agar to a depth of about 3 millimeters, being careful also to coat the sides of the funnel to a height that would be greater than the depth of the juice employed. Suction was then applied as before; filtration, however, was extremely slow. In the other case a cylindrical porous atmometer tube was partially filled with the melted agar, then by revolving the filter in a position almost horizontal and subsequently rapidly revolving it on a block of ice, as in the preparation of an Esmarch rolled plate, a layer of the agar was deposited throughout the length of the cylinder. In the case of the diffusion shells these were filled about half full with the diseased juice and then immersed to the depth of the inner liquid in small beakers of sterile distilled water. These were left for a period of 4 days at a temperature of about 18° C. in order that slow diffusion might proceed. The utmost care was used to prevent contamination of the exterior of any of the vessels employed. Inoculation experiments were made from each of the above tests, as indicated in the following outline.

TABLE I

INFECTION OF TOBACCO PLANTS WITH MOSAIC DISEASE AFTER FILTRATION OR DIFFUSION OF THE DISEASED JUICE

Experiment number	Nature of filter or diffusion shell	Source of infection	Number of plants diseased
1	Spherical atmometer cup	Filtrate	10
2	Control	Control (dt. water)	0
3	1.5% agar layer	Filtrate	0
4	3.0% agar layer	Filtrate	0
5	Parchment shell A	Liquid outside of tube	0
6	Parchment shell A	Liquid inside of tube	10
7	Parchment shell B	Liquid outside of tube	0
8	Parchment shell B	Liquid inside of tube	9

In the above experiments 10 tobacco plants were inoculated in each case. These were thrifty young plants of a common

variety, Kentucky burley. The inoculations were made on March 2 and final notes were taken March 16, though the plants were actually observed until April 11. No observations were made on temperature and humidity, but conditions in the greenhouse were such as to encourage rapid growth. These experiments were conclusive in showing that the virus or disease agency does not under our filtration conditions penetrate through agar of the consistencies employed, nor does it diffuse through a parchment membrane. On the other hand, the infected particles pass readily through the spherical atmometer cup. In this connection it should be observed that while the diffusion experiments lasted for a period of 4 days it has been shown (Allard, '16) that there is little, if any, lessening of pathogenicity in solutions subjected to more or less fermentation. The fact that the infected juice from within the diffusion shells invariably induced the disease is sufficient evidence that the growth of foreign organisms was not a factor worthy of consideration. The method of inoculation employed in the above experiments was the same as that described below for the more elaborate work here reported, and the reader is referred to the later description for the method employed.

It should be stated that several of the porous spherical atmometer cups have been tested in this laboratory under similar conditions and have been found invariably to prevent the passage of vegetative cells or of spores of *Bacillus subtilis*, and the subsequent results will show that this particular filter possesses finer pores than the Mandler diatomaceous filter. The indications furnished by Beijerinck as to the capacity of the virus to pass certain porous filters was again confirmed. On the other hand, Beijerinck claims a very slow diffusion, or penetration, of the virus into agar. The concentration of the agar is not noted. For the present the writers are unable to discuss the merits of this claim, since our own experiments represent direct filtration results, and the agar employed was probably denser than that used by Beijerinck.

FILTRATION OF THE DISEASED JUICE THROUGH ULTRAFILTERS

After the preliminary work reported above it was clear to the writers that it would be desirable to filter the diseased tobacco

juice through each of a series of porcelain or other filters of fairly well-determined porosity which might be subsequently standardized in a definite manner; but at the time no such series of filters could be found. Celloidin membranes did not seem to offer the range of porosity required. A little experimentation with rate of water flow, however, indicated that no inconsiderable range of possibilities was available in the form of the ordinary porcelain filters and atmometers of the laboratory. Accordingly, a series of filters was arranged consisting of a Mandler filter, a porous spherical atmometer cup, 2 cylindrical atmometer tubes, 2 cylindrical atmometer tubes infiltrated with precipitation films of $Al(OH)_3$, and 2 specially prepared celloidin membranes. Considerable preliminary work led to the selection of this series. It may be well also to indicate that the particular spherical atmometer cup used in this work proved to be the only one possessing pores noticeably finer than the average of these cups. This cup was one of the earlier ones distributed for work in atmometers.

Filters employed.—The porcelain filters were, where necessary, thoroughly cleaned and all were boiled in distilled water prior to use. The Mandler filter employed was No. 5090 of the Arthur H. Thomas catalog, $2\frac{1}{2}$–$5\frac{1}{8}$ inches, tested to 6–12 pounds air pressure without passing air bubbles. The cylindrical filters impregnated with $Al(OH)_3$ were prepared in the following manner: The filter tubes were filled with 5 per cent $AlCl_3$ and after allowing time for this to penetrate the walls thoroughly the tubes were suspended in beakers of 1 per cent NH_4OH until it appeared that the alkali had penetrated the cup, shown by a slight turbidity. The tubes were then carefully rinsed.

The celloidin membranes were prepared according to the method of Brown ('15) by which films of relatively great permeability can be obtained. The membranes were formed on the inside of beakers. An 8 per cent solution of Schering's celloidin in an equal volume of ether and absolute ethyl alcohol was poured into a beaker and allowed to drain over another beaker for 10 minutes. The beaker was then immediately immersed in distilled water. After about a minute the membrane was loosened from the sides of the beaker, washed in the water

for a short while, and allowed to dry over night at laboratory temperature. Since a very permeable membrane was desired, the film was put into 96 per cent ethyl alcohol for 24 hours at 20° C. and then thoroughly washed in water for a day. The films were cut into sizes large enough to fit over the broad end of a thistle tube. Tests of these membranes for leakage by the air bubble method were concurrent with the filtration experiments.

Preparation of the juice from diseased leaves.—A simple standard method, long in use in this laboratory for preparing the infected juice to be employed in experimental work, was adopted. This consists in pulping a known weight of the diseased leaves in a large mortar with a heavy pestle, then adding an equal weight of water and continuing the pulping until the leaf tissue is thoroughly crushed. The material is then filtered through cotton on a Buchner or ridged funnel. This diluted juice is used directly in the inoculation experiments.

Filtration of the juices.—In these experiments it was necessary to use every precaution possible to prevent accidental contamination of surfaces or vessels that might come in contact with the filtered juice. It was soon found that this could best be done by lowering the wet filter into the vessel containing the diseased juice to a suitable depth and then drawing the filtrate into the tube, rather than to draw the current from within outward. By the method indicated, as soon as sufficient filtrate had been drawn into the filter cup or tube, the filtration was stopped, and with sterile pipettes a quantity of the clear filtered juice was taken from within the cup and placed in clean vessels for use in inoculation.

With the various porcelain filters the water pump reduced the air pressure to 1/15–1/30 of an atmosphere. The filtrate was rapidly obtained in the case of the Mandler filter and also very nearly so rapidly in the case of the spherical atmometer cup. In fact, the time required to obtain a sufficient amount of the filtered juice was about 15 minutes with the spherical atmometer cup, and 30–45 minutes with the cylindrical ones. According to all the evidence at present available, such differences in pressure as were used do not materially influence the size of the

particles which may pass through, but primarily the rate of passage. The writers feel that it may be necessary to determine carefully the influence of the time interval; but since in these experiments comparative rather than fundamental results were desired, the phase of the filtration problem just referred to has not been experimentally studied.

With the celloidin membrane it was necessary to filter very cautiously so that a longer period of time at a pressure of 0.8 atmosphere was given. In this case, too, the membrane was fastened over the bell of a thistle tube. The diseased juice was then added through the tube, and the thistle tube—with the stem of the latter inserted through a rubber cork—was placed in a wide-mouthed bottle and lowered almost to the bottom, sufficient water being added to the bottle to just cover the membrane. Aspiration was then applied to the bottle through a second tube entering to just below the surface of the cork.

Inoculation Experiments with Filtered Juices

Technique of inoculation.—All inoculations were made by injuring the surface of the growing plant in 3 different areas, one from near the growing tip, one at the base of a young leaf, and another farther down the stem, or in the case of younger plants, just above the surface of the ground. These injuries were made with a needle or a fine pointed scalpel and in each case a drop of the infected juice was smeared over the injury and somewhat worked into it. This type of injury proved generally more effective than merely rubbing the stem or leaf as has been done in some cases. It was generally found advisable to make the inoculations in the late afternoon, the greenhouse being thoroughly watered afterward so as to prevent a too rapid drying of the injured surfaces.

Since there was some danger that the operator handling the filtration apparatus might come more or less in contact with particles of the diseased juice it was arranged that all inoculation work should be carried out by a different operator. Moreover, in most cases the different inoculation experiments were made by different operators. Where this was not possible every

precaution was taken with reference to contact with the clothing or hands. Between different inoculations the hands were washed with soap and water, then washed or rinsed with 1–500 formaldehyde, which has been found an effective antiseptic for the purpose, although when added to the juice in this concentration it is relatively ineffective.

Results of inoculation experiments.—There are given in table II the results of a series of inoculation experiments, with the filtered juices already described, conducted during November, 1921. In accordance with the indications previously given the inoculations were made on plants about 3 months old, which had been grown under greenhouse conditions and at this stage were in 5-inch pots. Good growing conditions were maintained throughout the experiment, since it has been repeatedly shown in our work that such conditions are favorable for most rapid production of unmistakable symptoms of the mosaic disease.

TABLE II

INOCULATION EXPERIMENTS MADE ON HEALTHY TOBACCO PLANTS, WITH FILTERED JUICES OBTAINED FROM PLANTS AFFECTED WITH THE CHARACTERISTIC MOSAIC DISEASE

Exp. No.	Number of plants	Source of the inoculation	No. of plants with mosaic after 18 days
1	20	Filtrate, Mandler filter	19
2	20	Filtrate, spherical atmometer cup	18
3	20	Filtrate, cylindrical atmometer tube A	1
4	20	Filtrate, cylindrical atmometer tube B	0
5	20	Filtrate, Atm. C. infilt. with $Al(OH)_3$	0
6	20	Filtrate, Atm. D. infilt. with $Al(OH)_3$	1†
7	20	Filtrate from celloidin membrane E	1†
8	—	Filtrate from celloidin membrane F*	—
9	20	Control, juice from diseased plant	19
10	20	Control, distilled water	0

* This membrane leaked and no inoculations were made.

† These two plants exhibited pronounced symptoms of mosaic in so short a time after inoculation that they are thought to have been accidental contaminations.

From the results obtained it was clear that particular interest would attach to the spherical atmometer cup and to the filter

of next lower porosity, which proved to be the cylindrical atmometer A. After the standardization was carried out, as discussed later, bearing out the importance of the work with these 2 cups a second series of inoculations was made with new filtrates of diseased juice through these 2 cups. Twenty tobacco plants were inoculated with each filtrate and numerous uninoculated controls were kept in adjacent plats. Between 10 and 18 days after the inoculations 19 plants developed the disease among those inoculated with the spherical cup filtrate and 5 plants became diseased from the filtrate of the cylindrical cup A. Thus the previous test was admirably confirmed and even better indications were afforded that a small number of infected particles pass the cylindrical atmometer cup.

Standardization of the Filters

In attempting to standardize the filters which had been employed in this work there was the possibility of using the same filter after a thorough cleansing, or the possibility of employing a similar filter assumed to be of equal porosity. It became evident that direct standardization of the original filter employed was essential where this could be done without fear of change or injury. Consequently the first step in the standardization involved a thorough cleansing of the filters employed. The standardization process was delayed until after the results of the inoculation in order to limit the amount of unnecessary work. From the inoculation experiments it was clear that the sizes of the infective particles must lie between the pore sizes of the spherical atmometer cup used and that of the most porous cylindrical tube A, and probably close to the pore sizes of the latter. At the time we had no idea of the relation of these sizes, and had not the subsequent standardization experiments indicated that these two pore sizes were sufficiently close together, it would have been necessary to seek further for a porous filter of intermediate pore dimensions.

To avoid difficulties arising from adsorption or from the possible action of electrolytes derived from the filters, it was determined to use organic sols rather than metallic sols for

standardization purposes. It was, however, with some regret that the use of gold sols was then considered undesirable, since the sizes of the particles in such solutions have been so well determined. In undertaking the standardization work it seemed best to use at the outset colloidal solutions that might represent extremes in sizes and then to narrow the field down to those that might correspond more nearly with the particles of the mosaic disease. Accordingly, a solution of dextrin was first used, since the particles represent extreme smallness in colloidal solutions, and moreover the filtrates could be readily tested by the simple iodine method. Filtration experiments with a 1 per cent dextrin solution indicated that these particles passed freely through all of the standard unimpregnated porcelain cups employed. A small quantity of dextrin passed the cylindrical cup C, impregnated with $Al(OH)_3$, and none passed the other cup so impregnated.

In the next test milk free of fat was employed with a view to determining the size relation between the mosaic disease particles and casein in milk. The milk was first filtered through the spherical atmometer cup and it was found that this filter prevents entirely the passage of casein. The filtrate was a clear solution containing no demonstrable quantity of casein. It was now necessary to utilize a larger colloidal molecule for standardization than dextrin, and yet a molecule considerably smaller than casein in milk, thus hemoglobin was selected.

The hemoglobin employed was a preparation made by standard methods from ox blood. As soon as the ox blood was drawn neutral potassium oxalate to make 0.2 per cent was added in order to prevent clotting. A measured quantity of the blood was then distributed in centrifuge tubes and centrifuged, the supernatant serum being drawn off and the known volume of corpuscles thoroughly washed 4 times with a physiological salt solution (0.9 per cent NaCl). An equal volume of distilled water was added to lake the corpuscles, after which the solution was again centrifuged to remove fibrin and stroma. The red supernatant colloidal solution was finally diluted so as to contain 1 per cent hemoglobin, estimating the original hemoglobin blood content at 12 per cent. For this work it was not necessary,

of course, to dialyze or otherwise further purify the product, as was requisite in the type of studies pursued by Bottazzi ('13), Reichert ('09), and others.

For ultrafiltration work hemoglobin has been recognized as a product of exceptional value. By any standard method of preparation it would seem that the particles are of fairly uniform size, so much so that it was employed by Bechhold in standardizing and designating the porosity of his gelatin filters. Nevertheless, the actual sizes of the particles do not seem to have been determined. In one of his papers Bechhold ('07) indicates that the particles must average a little less than 20 μμ, being fairly comparable with "Kollargol (koll. Silber v. Heyden)". In his text ('19), moreover, the same author places them at smaller than the particles of 1 per cent gelatin and larger than serum albumen, which would indicate a measurement somewhat greater than 30 μμ. Later in the same work (p. 111) he indicates the sizes of hemoglobin particles at 33–36 μμ. The diameter of the hemoglobin molecule has been given as 2.3–2.5 μμ.

The tests with the standardized hemoglobin solution yielded results both satisfactory and illuminating. Through the Mandler filter with the usual time interval mentioned the filtrate was a very deep red, yielding no appreciable dilution of the hemoglobin. Through the spherical atmometer cup the filtrate was still very red, indicating that relatively few particles of the hemoglobin were held back. Through the cylindrical atmometer cup A there was a very slight passage of hemoglobin particles, while through the cylindrical tube B and both tubes impregnated with $Al(OH)_3$ there was no passage of hemoglobin particles whatsoever in these tests.

Further it may be of interest to state that the spherical atmometer cup referred to above permitted approximately 50 per cent of the gelatin particles to pass fhrough the filter from a 1 per cent solution of gelatin. The amount passing through was determined colorimetrically in comparison with the original solution by means of the Biuret test. The gelatin solution was prepared by adding gelatin to the boiling water and then immediately cooling to room temperature.

DISCUSSION

From the results presented it would seem clear that with approximately equal pressures and equal time intervals the infective particles of the juice of tobacco plants affected with the mosaic disease possess about the same capacity to pass through the pores of porcelain filters as do the colloidal particles of fresh hemoglobin prepared by standard methods. No determinable dilution or loss of infectivity of the tobacco juice was occasioned by filtration through the spherical atmometer cup used in these experiments. On the other hand, a dilution of approximately 50 per cent resulted when a 1 per cent gelatin solution was filtered in the same cup. The sizes of the infective particles would therefore appear to be considerably less than those of gelatin particles, and since the particles of gelatin are not apparently very much larger than those of hemoglobin the conclusion is further strengthened that the infective particles here in question have about the size relations of fresh hemoglobin. In considering the estimated size of hemoglobin particles referred to previously in connection with the work of Bechhold it should be pointed out that Bechhold seems to have worked with dried preparations of hemoglobin, and it is perhaps to be expected that these would be larger rather than smaller than those of the fresh product. All indications are that, in general, a relatively freshly made colloidal solution possesses particles more uniform in size, and this idea is tentatively accepted. Assuming that at most the hemoglobin particles worked with may have possessed a diameter of 30$\mu\mu$, more or less, and that the average small diameter of bacterial plant pathogens is around 1000$\mu\mu$ (some being as low as 500 and others as large as 1500$\mu\mu$) we have 30: 1000 to express roughly the diameter relations of mosaic disease particles in comparison with bacterial plant pathogens. On the basis of this average relation it is interesting to note that the volume relation would be about as follows: 1:37,000, or about 26: 1,000,000, assuming that in each case we may treat the bodies as spherical structures.

The results of the filtration experiments have directed the attention of the writers to the possibility of the existence of

minute organisms or propagative parts of organisms in the soil or in other products which are commonly the seat of varied bacterial activities. While this has been previously pursued in certain directions an investigation of one important aspect of the problem has been undertaken. This work will be reported upon in a subsequent paper.

No reference has thus far been made to two recent reports by Kunkel which are of particular interest in this connection. In the earlier paper Kunkel ('21) has studied cytologically the tissues of corn affected with a mosaic disease and he reports, describes, and figures a foreign body believed to be a living organism invariably present in the diseased cells. The distribution of this body is found to correspond with the distribution of the light green color areas in the diseased leaves. While no proof has been afforded that these bodies are etiologically related to the corn mosaic, or even that they are living structures, it is suggested that they may be more or less analogous to the Negri bodies in the brain cells of animals suffering from rabies. In a more recent note Kunkel ('22) has associated ameboid bodies with the *Hippeastrum* mosaic, this host plant being a member of the *Amaryllidaceae*. Analogous bodies have not thus far been mentioned by those who have studied the mosaic disease of tobacco histologically or cytologically.

It is of course not certain that the mosaic diseases of these monocotyledonous plants are caused by organisms or agencies similar to those inducing the mosaic of tobacco. At the present time either possibility may be entertained. Even should an ameboidal structure be found in the cells affected with mosaic disease of tobacco and etiologically associated therewith, interest in the filtration experiments would remain. Whatever might be the size relations of such an organism in the uninjured cell, its behavior under filtration would indicate that relatively minute colloidal particles of the body are capable of reproducing it. A discussion of theoretical aspects is reserved until further experimental work has been done.[1]

[1] Since the above was written the attention of the writers has been drawn to an article previously overlooked on a filterable virus, as follows: Andriewski, P. L'ultra-filtration et les microbes invisibles. Centralbl. f. Bakt. I. **75:** 90–93. 1914. Using

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the virus of the "peste des poules," this investigator compared the ultrafiltration of this disease agency with hemoglobin and serum albumin, all filtrations being made through a graded series of collodion membranes. It was determined that the particles of the virus were smaller than hemoglobin and about the size or somewhat smaller than serum albumin. In discussing actual sizes, however, he seems to confuse the sizes of colloidal particles of hemoglobin with the sizes of molecules. Nevertheless, his conclusion is to the effect that this virus cannot be formed of cells similar to those of plants and animals at present known.